

**Retinoic acid receptor activity is required for the maintenance of type 1
innate lymphoid cells**

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Group 1 innate lymphoid cells (G1-ILCs) are innate immune effectors critical for the response to intracellular pathogens and tumors. G1-ILCs comprise circulating natural killer (NK) cells and tissue-resident type 1 ILCs (ILC1s). ILC1s mainly reside in barrier tissues and provide the initial sources of interferon- γ (IFN- γ) to prime the protecting responses against infections, that are followed by the response of recruited NK cells. Despite such distribution differences, whether local environmental factors influence the behavior of NK cells and ILC1s is unclear. Here, we show that the signaling of retinoic acid (RA), active metabolites of vitamin A, is essential for the maintenance of ILC1s in periphery. Mice expressing RAR α 403, a truncated form of retinoic acid receptor α (RAR α) that exerts dominant negative activity, in a lymphoid cell- or G1-ILC-specific manner showed remarkable reductions of peripheral ILC1s while NK cells were unaffected. Lymphoid cell-specific inhibition of RAR activity resulted in reduction of PD-1⁺ ILC progenitors (ILCPs), but not of common lymphoid progenitors (CLPs), suggesting the impaired commitment and differentiation of ILC1s. Transcriptome analysis revealed that RAR α 403-expressing ILC1s exhibited impaired proliferative states and declined expression of effector molecules. Thus, our findings demonstrate that cell-intrinsic RA signaling is required for the homeostasis and the functionality of ILC1s, which may present RA as critical environmental cues targeting local type 1 immunity against infection and cancer.

Introduction

Group 1 ILCs (G1-ILCs) play vital roles in response to intracellular infections and tumors (1,2). G1-ILCs contain circulating natural killer (NK) cells and tissue-resident type 1 ILCs (ILC1s), with similar features such as the expression of surface NK1.1 and NKp46, transcription factor T-bet, and production of abundant interferon- γ (IFN- γ) (1,3,4). In contrast, murine ILC1s are generally distinguished from NK cells by expression of CD49a as well as lack of CD49b and transcription factor Eomes (5-7). The developmental trajectory of ILC1s also clearly differ from NK cells, as evidenced by the fact that common lymphoid progenitors (CLPs) can generate both NK cells and ILC1s while PLZF⁺PD-1⁺ ILC progenitors (ILCPs), a progeny of CLPs, are committed to ILC1s, ILC2s, and ILC3s but not to NK cells (8-10). Recent studies have also shed light on the unique ILC1 roles as the early interferon- γ (IFN- γ) producers and the local mediators of type 1 immune response during virus infection and liver injury (11-13). Another important feature of ILC1s is heterogeneity among tissues; NK cells are observed as relatively uniform populations through the body whereas ILC1s represent highly diverse surface molecule expression and transcription factor requirements across organs (7,14,15), suggesting that tissue-specific cues play key roles in ILC1 regulation. However, how local environmental factors differentially regulate NK cells and ILC1s to maintain their pool size and the proper functionality is unclear.

Retinoic acid (RA), highly bioactive metabolites of vitamin A, is essential for various biological processes such as embryonic development, epithelial integrity, and immune function (16,17). Vitamin A deficiency is associated with increased various infectious diseases such as diarrhea, respiratory infections including measles, and human immunodeficiency virus (HIV) infection (18,19). Mechanistically, RA locally produced by retinaldehyde dehydrogenases (RALDH)-expressing cells binds to target cell retinoic acid receptors (RAR α , β , and γ), that form heterodimers with retinoid X receptors (RXRs). RAR/RXR complexes bind to RA response elements (RAREs) to directly modulate expression of vast target genes including immune-related genes such as *Nfatc1* (20), *Il22* (21), *Il9* (22), and *Rorc* (23). RA thereby regulates T cell function, localization, and effector differentiation in the intestine (17,24). RA is also involved in ILC regulation: promoting the migration and development of ILC3s and inhibiting ILC2 development in the intestine (23,25-27). However, despite the well-known relevance between RA and infection prevention, how RA influences NK cells and ILC1s still remains unknown.

81 To address this question, we examined the lymphoid cell- and G1-ILC-intrinsic
82 requirements for RAR activity in vivo by using mice carried RAR α 403 transgenes, a
83 truncated form of RAR α which can bind to ligands but no longer modulates transcription (28),
84 in the Rosa26 locus (Rosa26-RAR α 403/+ mice) (29). Mice in which RA signaling is
85 specifically inhibited in lymphoid cells (Il7r-Cre Rosa26-RAR α 403/+ mice) lacked ILC1s, in
86 contrast to unaffected NK cells, in the liver and mesenteric lymph nodes (mLNs). In addition,
87 ILCPs were decreased in Il7r-Cre Rosa26-RAR α 403/+ mice, suggesting that commitment and
88 differentiation of ILCs depend on RAR activity. Moreover, we demonstrated that ILC1s
89 depended on RA signaling in a cell-intrinsic manner by using Ncr1-Cre Rosa26-RAR α 403/+
90 mice, in which RAR activity is ablated specifically in G1-ILCs. Transcriptome analysis
91 showed the impaired proliferative and functional signatures in ILC1s of Ncr1-Cre Rosa26-
92 RAR α 403/+ mice. Thus, our findings reveal that RA signaling is critical for the homeostasis
93 and functionality of peripheral ILC1s.

Methods

Mice

C57BL/6 mice were purchased from Japan SLC (Hamamatsu, Japan). Six to twelve-weeks old mice on a C57BL/6 background were analyzed. Rosa26-RAR α 403/+ mice (29) were kindly provided by Dr. Cathy L. Mendelsohn (Columbia University, USA) and crossed with either Ncr1-Cre transgenic (Tg) mice (30) provided by Dr. Veronika Sexl or Il7r-Cre knock-in (KI) mice (31) provided by Dr. Hans-Reimer Rodewald. All mice were maintained under specific pathogen-free conditions in the Experimental Research Center for Infectious Diseases in the Institute for Life and Medical Sciences, Kyoto University. All procedures were carried out under sevoflurane or isoflurane anesthesia to minimize animal suffering. All mouse protocols were approved by the Animal Experimentation Committee of the Institute for Life and Medical Sciences, Kyoto University.

Cell preparation and isolation

Liver and mLNs were dissociated mechanically and passed through 70- μ m cell strainers (Greiner Bio-One, Milan, Italy). Adult liver leukocytes were then separated by centrifugation through 40% Percoll. Bone marrow (BM) cells were obtained by flushing out the marrow fraction of femurs and tibias using a syringe with a 27 G needle (Terumo Corporation, Tokyo, Japan).

Flow cytometry and cell sorting

Flow cytometry and cell sorting were performed on BD FACSVerse or BD LSRFortessa X-20 flow cytometers (BD Biosciences) and BD FACS Aria II or Aria III cell sorters (BD Biosciences), respectively. Data were analyzed on FlowJo software (FlowJo, Ashland, OR, USA). Debris and dead cells were excluded from analysis by forward and side scatter and propidium iodide (PI) gating. In figures, values in quadrants, gated areas, and interval gates indicate percentages in each population. For antibody staining, following fluorescent dye- or biotin-conjugated antibodies were used: CD3 ϵ (145-2C11), NK1.1 (PK136), NKp46 (29A1.4), CD49a (HM α 1), CD49b (DX5), IL-7R α (A7R34), F4/80 (BM8), Gr-1 (RB6-8C5), CD19 (6D5), B220 (RA3-6B2), TCR β (H57-597), Fc ϵ RI (MAR-1), PD-1 (29F.1A12), α 4 β 7 (DATK32), Flt3 (A2F10), CD25 (PC61), Ki-67 (SolA15), Bcl-2 (BCL/10C4), and ROR γ t (Q31-378) (BioLegend, San Diego, CA, USA; Thermo Fisher Scientific, Waltham, MA,

USA; BD Bioscience, San Jose, CA, USA; TONBO Biosciences, San Diego, CA, USA). Early apoptosis of NK cells and ILC1s was detected using MEBCYTO Apoptosis Kit (MBL Life Science, Nagoya, Japan), and live annexin V⁺ cells were termed as “Annexin V⁺ cells”. Biotinylated monoclonal antibodies were detected with Brilliant Violet 421-conjugated streptavidin (Thermo Fisher Scientific).

RNA sequencing (RNA-seq) and data analysis

NK cells (CD49a⁻CD49b⁺) and ILC1s (CD49a⁺CD49b⁻IL-7R⁺) were freshly sorted (2×10^2 cells) from control or Ncr1-Cre Rosa26-RAR α 403 mice, lysed with Buffer RLT (Qiagen, Hilden, Germany), and purified with RNAClean XP (Beckman Coulter, Brea, CA, USA). Double strand cDNA was synthesized, and sequencing libraries were constructed using SMART-seq HT Plus kit (Takara Bio, Otsu, Japan). Sequencing was performed with 150 bp paired-end reads on the Illumina HiSeq X (Illumina, San Diego, CA, USA) sequencer. fastp (32) was used to assess sequencing quality and to exclude low-quality reads and adaptor contaminations. Reads were mapped on the mouse reference genome (mm10) using HiSat2. The read counts were determined at the gene level with featureCounts. Normalization of gene expression levels and differential gene expression analysis were performed using DESeq2. Genes were considered as differentially expressed genes (DEG) when they had an adjusted p (p_{adj}) value < 0.3. Metascape (33) was used for enrichment analysis.

Statistical analysis

Statistical differences were evaluated by the two-tailed unpaired Student's *t*-test and one-way analysis of variance (ANOVA) using GraphPad Prism 8 (GraphPad Software, San Diego, California, USA). Asterisks in all figures indicate as follows: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$.

Data availability

The accession number for RNA-seq data of G1-ILCs in control or Ncr1-Cre Rosa26-RAR α 403 mice generated in this study is deposited in Gene Expression Omnibus (GEO): GSE205895.

Results and discussion

Lymphoid cell-specific inhibition of RAR activity depletes ILC1s

Vitamin A and RA promote ILC3 development whereas suppress the population sizes of ILC2s (23,26,27). Although the overall cellularity of NK1.1⁺ G1-ILCs are unaffected by RA signaling inhibition (26,27), how RA impacts NK cells and ILC1s individually remains unclear. To address this question, we generated Il7r-Cre Rosa26-RAR α 403/+ mice (Il7r-Cre^{RAR α 403} mice) (29), in which RAR activity was inhibited in all lymphoid lineage cells (lymphoid lineage precursors such as CLPs, conventional and unconventional T cell and B cell lineages, and all ILCs and ILCPs) (31) in a dominant negative manner. In flow cytometry (FCM) analysis, NK cells and ILC1s were identified as CD49a⁻CD49b⁺ and CD49a⁺CD49b⁻ populations, respectively, within CD3⁻NK1.1⁺NKp46⁺ G1-ILCs in the liver and mLNs (Fig. 1A and 1B). In both Il7r-Cre Rosa26-+/+ (Il7r-Cre^{WT}) control mice and Il7r-Cre^{RAR α 403} mice, CD3⁺ T cells and CD19⁺ B cells were normally developed (Fig. 1C and 1D). NK cells were also unchanged in Il7r-Cre^{RAR α 403} mice (Fig. 1E). Surprisingly, Il7r-Cre^{RAR α 403} mice mostly lacked ILC1s in the liver and mLNs (Fig. 1F). In addition, CD3⁺NK1.1⁺ NKT cells were also reduced in Il7r-Cre^{RAR α 403} mice (Fig. 1G). These results show that RAR activity impacts the homeostasis of innate-like lymphocytes in a subset-dependent manner and is especially critical for the presence of ILC1s.

RAR activity is involved in the ILC commitment and differentiation

To address the effects of RAR activity in ILC development, we examined whether RAR α 403 expression impacts the precursor fractions of ILCs, such as CLPs (Lin⁻IL-7R⁺FLT3⁺) and ILCPs (Lin⁻IL-7R⁺FLT3⁻ α 4 β 7⁺PD-1⁺) (34) (Fig. 2A). The frequency of ILCPs decreased in BM of Il7r-Cre^{RAR α 403} mice compared to controls, whereas the frequency of CLPs was slightly increased ($p = 0.064$) (Fig. 2B). We next analyzed the ILCP progenies other than ILC1s. Consistent with previous studies (23,26), the cell number of ILC3s (Lin⁻IL-7R⁺ROR γ t⁺) were decreased in mLNs of Il7r-Cre^{RAR α 403} mice (Fig. 2C). In addition, ILC2s (Lin⁻IL-7R⁺FLT3⁻CD25⁺) were also reduced in BM and mLNs of Il7r-Cre^{RAR α 403} mice (Fig. 2D), suggesting that the effect of systemic vitamin A deficiency and treatment of RA antagonists examined so far, that suppress the ILC2 number (27,35), may differ from that of cell-intrinsic inhibition of RA signaling. It is also possible that occupancy of RXRs by RAR α 403 might exert unexpected effects on the function of other RXR partners, such as

VDR and PPARs (36,37), although minimal levels of expression of these genes were detected at least in NK cells and ILC1s (data not shown). Collectively, these results show that RAR activity is required for ILCPs but not CLPs, suggesting that RA promotes commitment and differentiation of ILCs but not overall development of lymphocytes.

ILC1s require RA signaling for their homeostasis in a cell-intrinsic manner

To address the G1-ILC-intrinsic requirement for RAR activity, we analyzed Ncr1-Cre Rosa26-RAR α 403/+ mice (Ncr1-Cre^{RAR α 403} mice) compared with Ncr1-Cre Rosa26-+/+ (Ncr1-Cre^{WT}) controls. As expected, T cells and NKT cells were unchanged in Ncr1-Cre^{RAR α 403} mice (Fig. 3A and 3B), consistent with specific G1-ILC targeting of Ncr1-Cre mice (30). Notably, ILC1s, but not NK cells, were significantly reduced in the liver of Ncr1-Cre^{RAR α 403} mice (Fig. 3C and 3D). Similar trends were observed in the small intestine and the spleen (data not shown). These results demonstrate the cell-intrinsic requirement for RAR activity in ILC1s across organs.

RA signaling maintains the proliferative state and functionality of ILC1s.

An ILC1-restricted reduction in Ncr1-Cre^{RAR α 403} mice indicates that RA signaling directly modulates the cellular state of mature ILC1s. To address the RA signaling-mediated effects in detail, we conducted bulk RNA sequencing (RNA-seq) experiments on liver NK cells, liver ILC1s, and spleen ILC1s freshly sorted from control or Ncr1-Cre^{RAR α 403} mice. In RNA-seq experiments, liver and spleen ILC1s were identified as CD49a⁺CD49b⁻IL-7R⁺ G1-ILCs to achieve further purification of ILC1s (9) (Supplementary Figure S1A). Genes significantly downregulated in Ncr1-Cre^{RAR α 403} mice compared to controls (down-DEGs) were detected and calculated in each G1-ILC population: 8 genes for NK cells, 56 genes for liver ILC1s, and 42 genes for splenic ILC1s (Fig. 4A and Supplementary Figure S1B), confirming the larger impacts of RAR activity against ILC1s than NK cells. Enrichment analysis using Metascape (33) revealed that both down-DEGs of liver and splenic ILC1s were enriched with the pathways related to proliferation and cell cycle (Fig. 4B, 4C, and 4D), whereas down-DEGs of NK cells had no enriched pathway. Consistent with this, *Mki67* gene and Ki-67 protein expression were reduced in liver ILC1s in Ncr1-Cre^{RAR α 403} mice (Fig. 4E and 4F). We found no change in their Bcl-2 expression and the frequency of Annexin V⁺ cells (Fig. 4G and 4H), suggesting that the ILC1 survival are intact in Ncr1-Cre^{RAR α 403} mice. These data suggest that RA activity supports the proliferation of ILC1s.

223 Interestingly, down-DEGs of liver ILC1s contained several chemokines (*Ccl3*, *Ccl4*,
224 and *Xcl1*) (Fig. 4I), suggesting the impaired functionality. Since ILC1s localize in the
225 frontline of infection and play a critical role in the priming of type 1 immune response (11), it
226 is possible that ILC1s are involved in leukocyte recruiting or directly suppress viruses via
227 these chemokines (38). In addition, five genes were identified as the intersection of down-
228 DEGs in liver and splenic ILC1s (Fig. 4J), in which *Egr1* was the most variant one. RARs
229 directly bind to RARE at the 5'-proximal region of *Egr1* and promote its expression (39-41).
230 EGR1 has been reported to enhance chemokine expression including CCL3 and CCL4 (42-
231 44). Thus, these results suggest the possibility that RAR activity promotes chemokine-
232 mediated ILC1 functions via direct induction of EGR1.

233 Accumulating evidence has shown that RA is a critical niche factor regulating
234 development, maintenance, and function of immune cells such as intestinal induced Tregs
235 (iTregs) (45) and peritoneal macrophages (46). Given that, it is possible that RA-producing
236 cells across multiple tissues provide niches for ILC1s, which locally maintain ILC1 pool size
237 and proper functionality to immediately respond to infection. Therefore, it would be
238 increasingly critical to determine the precise RA source for ILC1s. In the liver, RALDH
239 expression is detected in hepatic stellate cells (47), liver sinusoidal endothelial cells (LSECs)
240 (48), and hepatocytes (49), although their roles in vivo are not determined. Future
241 investigations addressing the actual RA source for ILC1s will lead to further understanding of
242 the mechanism of local immune regulation. Taken together, our findings have presented RA
243 as a possible key regulator for the local type 1 immunity and provide mechanistic insights into
244 the critical roles of vitamin A in infection prevention.

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Figure legends

Fig. 1. Lymphoid cell-specific inhibition of RAR activity depletes ILC1s but not NK cells.

(A–G) Flow cytometric (FCM) analysis of liver and mLN lymphocytes in Rosa26-RAR α 403/+ mice, Il7r-Cre^{WT} mice, or Il7r-Cre^{RAR α 403} mice. Representative FCM profiles in the liver (A) and mLNs (B), the percentages (*upper*) and the cell numbers (*lower*) of T cells (C), B cells (D), NK cells (E), ILC1s (F), and NKT cells (G) in indicated tissues are shown. Data represent two to three independent experiments (n = 4–6). Data are presented as mean $\pm$ SEM. * p < 0.05, ** p < 0.01, **** p < 0.0001.

Fig. 2. RA signaling is required for development of ILCPs.

(A–D) FCM analysis of CLPs, ILCPs, ILC3s, and ILC2s in Il7r-Cre^{WT} (control) or Il7r-Cre^{RAR α 403} mice. Representative FCM profiles in BM (A) and the percentages (*upper*) and the cell numbers (*lower*) of CLPs and ILCPs (B), ILC3s (C), and ILC2s (D) in indicated tissues are shown. Data represent two to three independent experiments (n = 3–6). Data are presented as mean $\pm$ SEM. * p < 0.05, ** p < 0.01, **** p < 0.0001.

Fig. 3. Cell-intrinsic RAR activity is required for maintenance of peripheral ILC1s.

(A–D) FCM analysis of liver and mLN lymphocytes in Ncr1-Cre^{WT} or Ncr1-Cre^{RAR α 403} mice. Representative FCM profiles in the liver (A), the percentages of T cells and NKT cells (B), and the percentages (*upper*) and the cell numbers (*lower*) of NK cells (C) and ILC1s (D) in the liver and mLNs are shown. Data represent two to three independent experiments (n = 3–5). Data are presented as mean $\pm$ SEM. * p < 0.05.

Fig. 4. RA signaling supports the proliferative and functional statuses in ILC1s.

(A) Number of genes significantly upregulated (blue; up-DEGs) and downregulated (red; down-DEGs) in each cell population of Ncr1-Cre^{RAR α 403} mice compared to Ncr1-Cre^{WT} mice are shown. (B and C) Dot plots showing the enriched pathways on down-DEGs of liver (B) and spleen (C) ILC1s. Genes count indicates the number of DEGs included in the pathway. Gene ratio is the ratio of genes count to the total gene number in the pathway. (D) Heatmap representing normalized expression levels of the genes related to cell cycle in liver ILC1s from Ncr1-Cre^{WT} (control) or Ncr1-Cre^{RAR α 403} mice followed by range scaling. (E) Normalized read counts of *Mki67* (p_{adj} = 0.254) expressed in liver ILC1s from control or

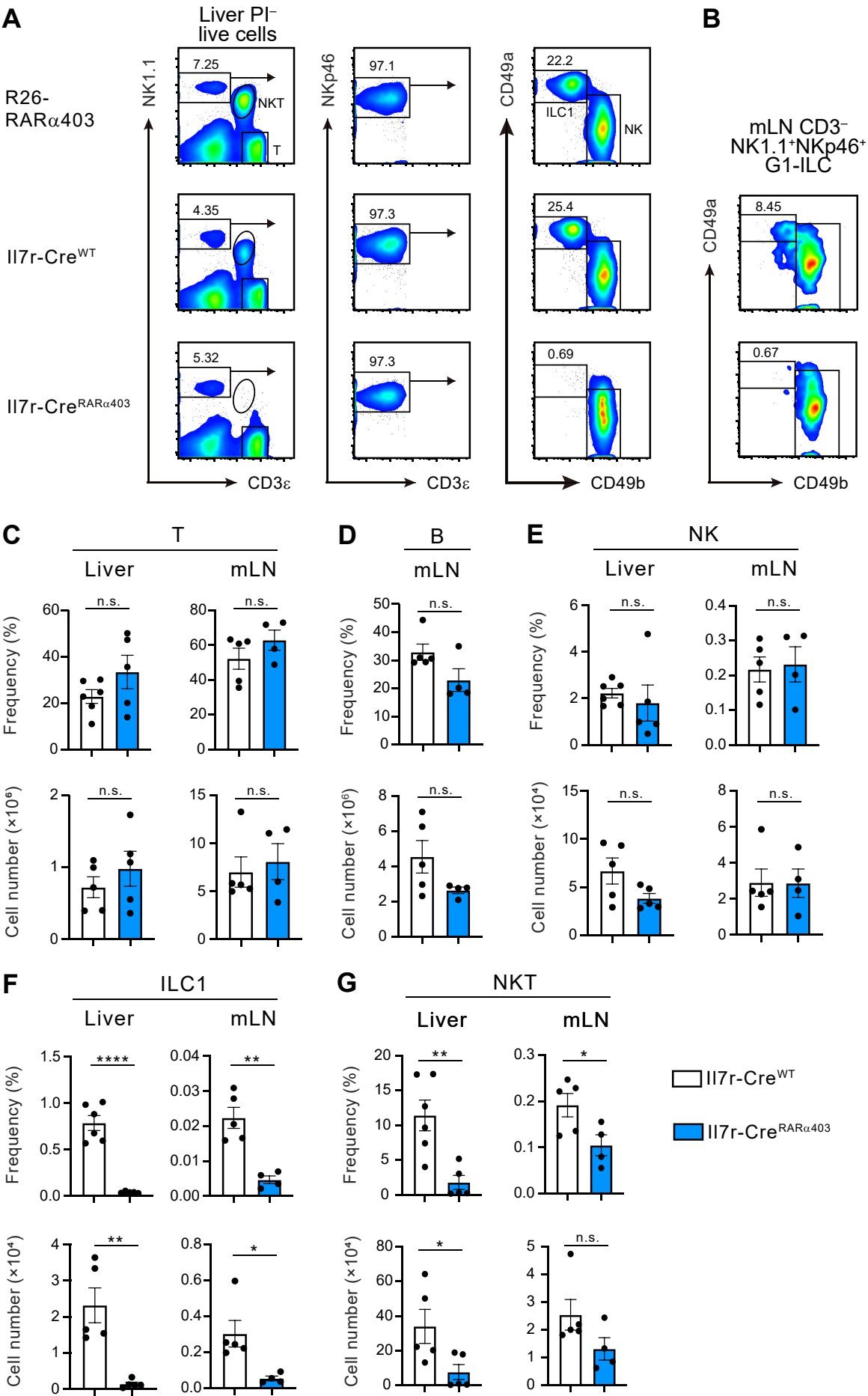
444 Ncr1-Cre^{RAR α 403} mice. **(F–H)** FCM analysis of proliferation and survival marker expression in
445 liver NK cells and ILC1s of Ncr1-Cre^{WT} or Ncr1-Cre^{RAR α 403} mice. The percentages of Ki-67⁺
446 cells (F), MFI levels of Bcl-2 (G), and the percentages of Annexin V⁺ cells (H) are shown. **(I)**
447 Normalized read counts of *Ccl3* ($p_{adj} = 0.013$), *Ccl4* ($p_{adj} = 0.203$), and *Xcl1* ($p_{adj} = 0.078$)
448 expressed on liver ILC1s from control or Ncr1-Cre^{RAR α 403} mice. **(J)** Venn diagram showing
449 the overlap between down-DEGs of liver and spleen ILC1s. Data represent two independent
450 experiments (F–H; n = 4–5) or are from RNA-seq experiments with three biological replicates
451 (A–E, I, and J). Data are presented as mean $\pm$ SEM. * $p < 0.05$.

Supplementary figure legends

Supplementary Figure S1. Transcriptome analysis of G1-ILCs in Ncr1-Cre^{RARα403} mice

(A) IL-7R expression of liver (*upper*) and spleen (*lower*) CD49a⁺CD49b⁻ G1-ILCs in Ncr1-Cre^{WT} or Ncr1-Cre^{RARα403} mice. (B) Volcano plots showing gene expression of each G1-ILC population in Ncr1-Cre^{WT} (control) mice relative to that in Ncr1-Cre^{RARα403} mice. Genes significantly downregulated (red; down-DEGs) or upregulated (blue; up-DEGs) in each cell population of Ncr1-Cre^{RARα403} mice are highlighted. *p*_{adj}, adjusted *p* value. FC, fold change. Data represent three (liver) and one (spleen) experiments (A) or are from RNA-seq experiments with three biological replicates (B).

Figure 1



A

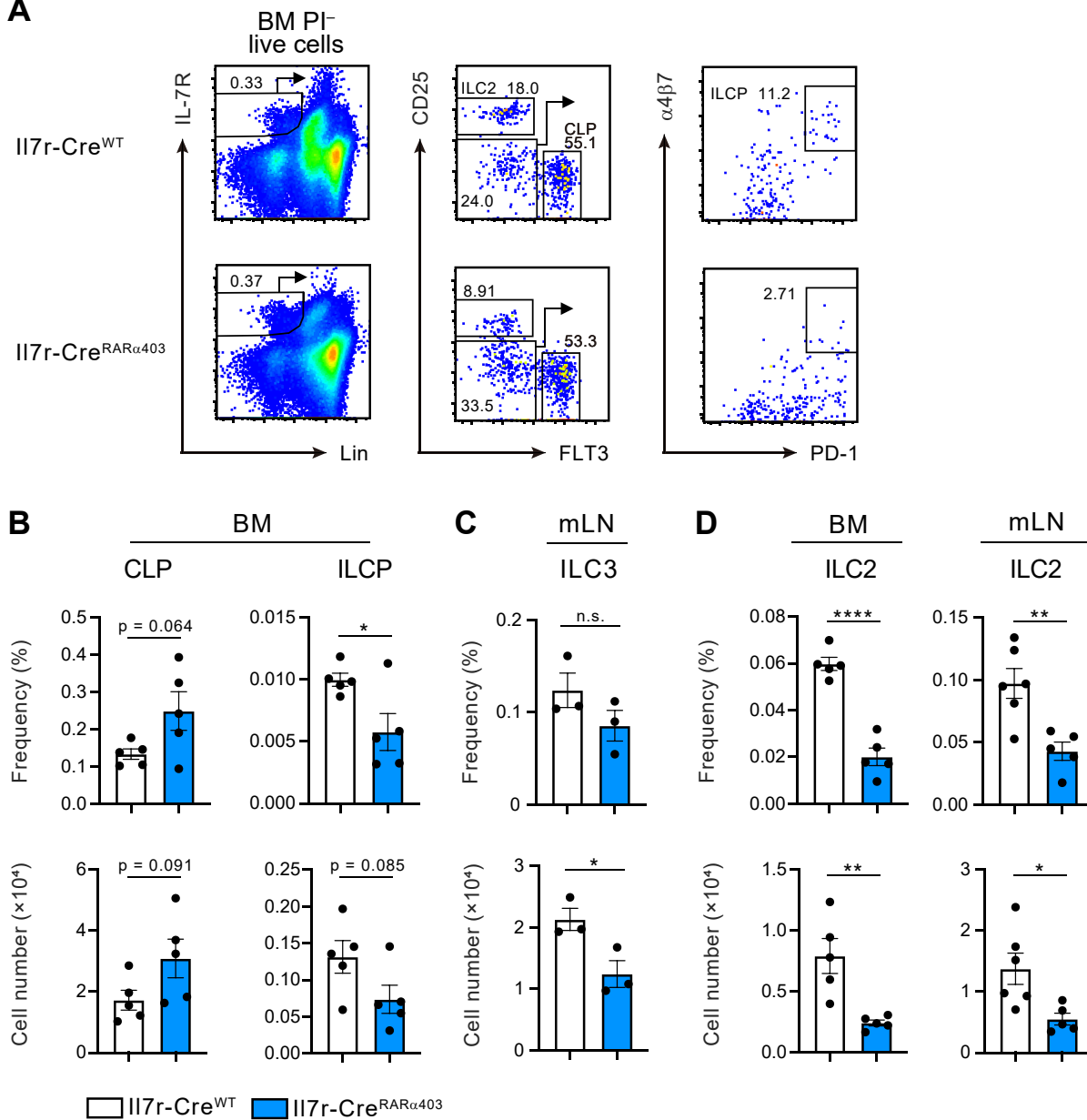


Figure 3

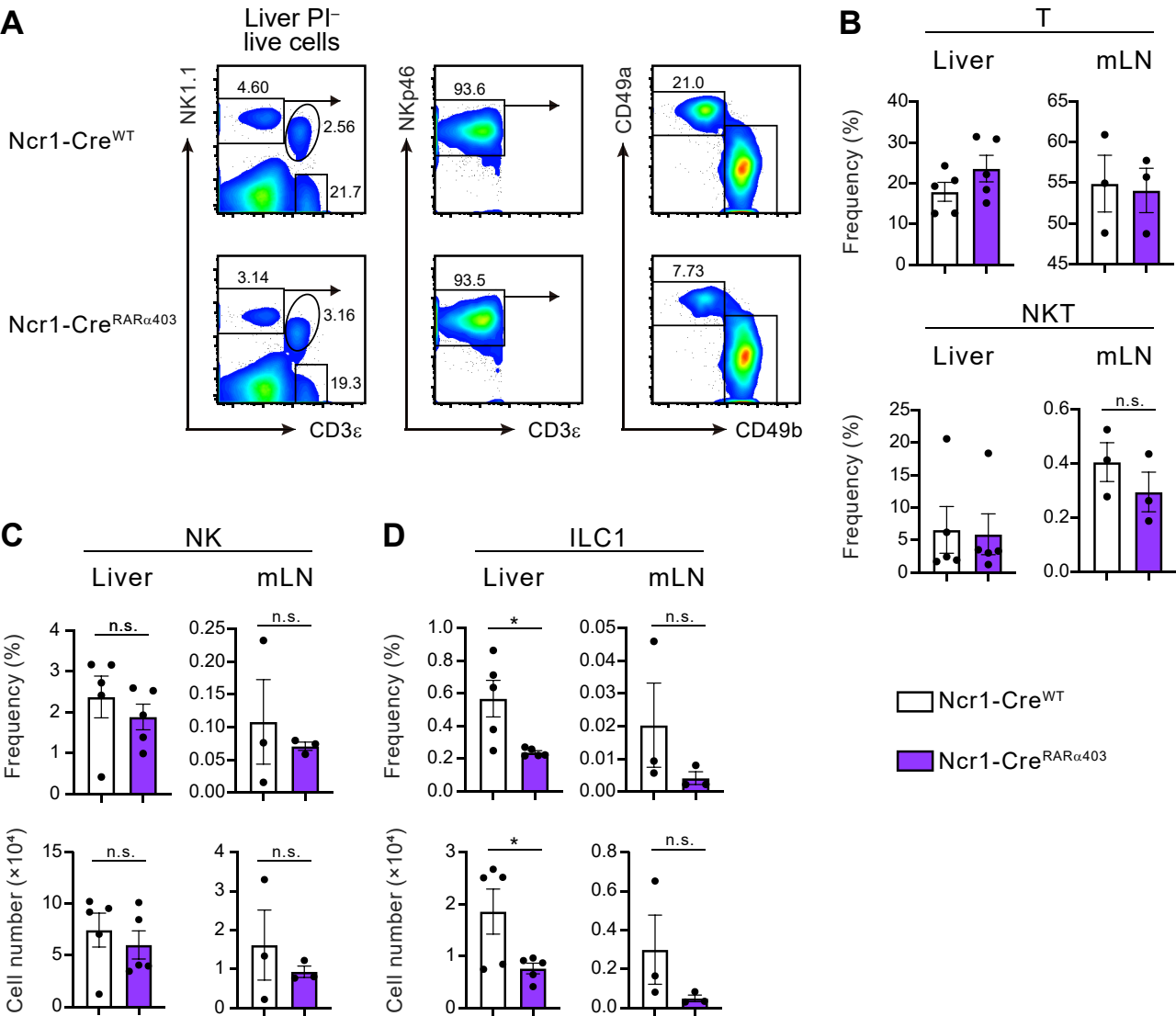
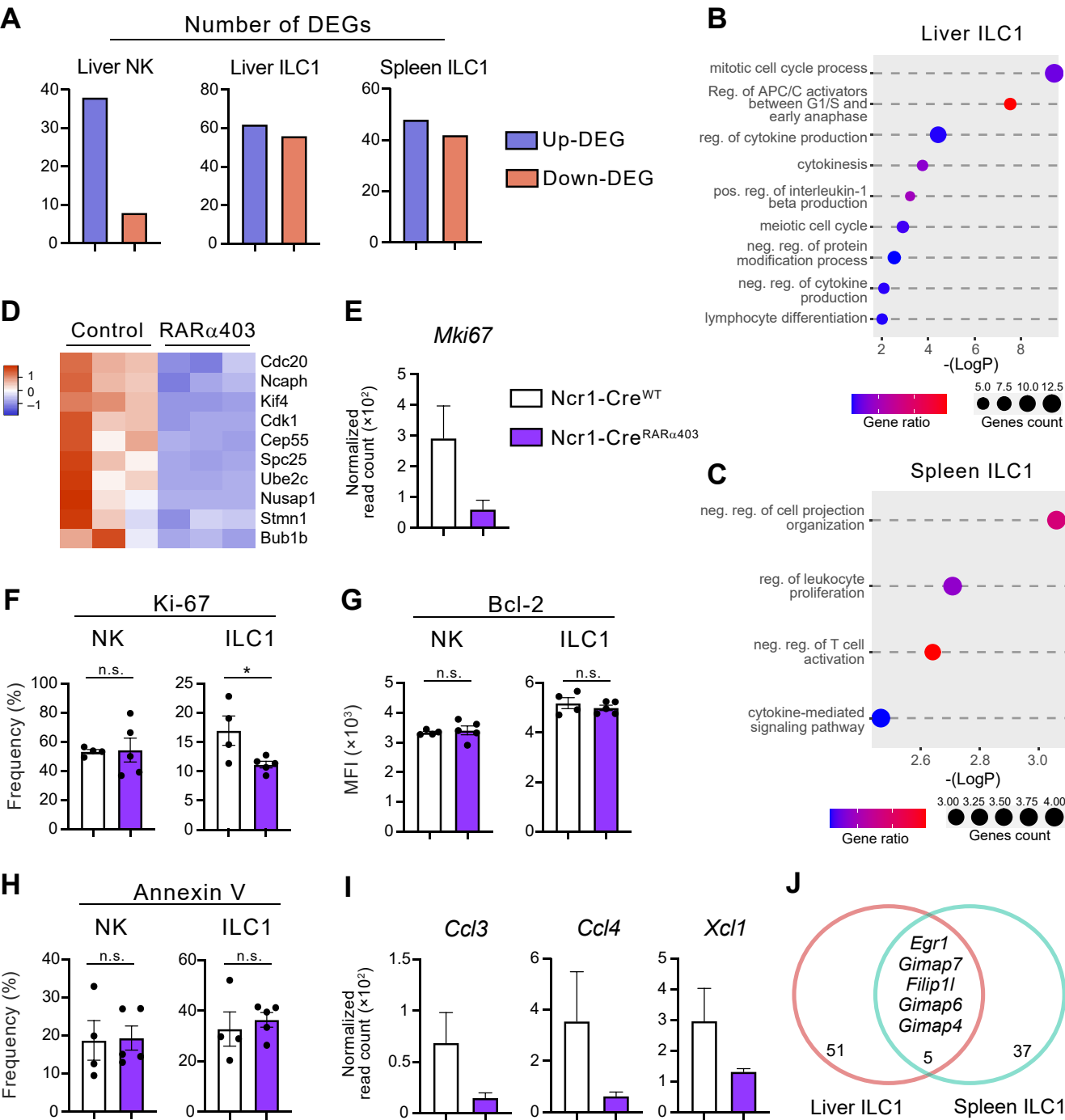


Figure 4



Supplementary Figure S1

